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The impact of some microcystins on the growth of heterotrophic bacteria from Portuguese freshwater reservoirs

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ABSTRACT

The impact of some microcystins on the growth of heterotrophic bacteria from Portuguese freshwater reservoirs

Microcystins (MCs) are hepatotoxins that are abundantly produced by cyanobacteria. Studies have shown that these toxins affect many multicellular organisms that inhabit aquatic ecosystems; however, their impact on bacteria that co-occur with freshwater cyanobacteria is still unclear.

In this study, the impact of the three most common variants of MCs (MCLR, MCRR, and MCYR) on the growth of heterotrophic bacteria isolated from four Portuguese reservoirs was evaluated. Some isolates were derived from freshwater sources where blooms of cyanobacteria that often contain microcystin-producing strains are frequently observed and from a reservoir where these phenomena do not occur. Morphological and molecular characterisation of the bacterial isolates was performed, and these bacteria were exposed to three different concentrations of each MC variant. The effect of MCs on bacterial growth was then evaluated.

This study showed that MCLR, MCRR and MCYR can reduce the growth of some heterotrophic bacteria isolated from freshwater sources. To our knowledge, this is the first study in which the impact of several variants of MCs was evaluated in a diverse group of freshwater heterotrophic bacteria.

Key words: Microcystin-LR, microcystin-RR, microcystin-YR, heterotrophic bacteria, growth inhibition.

RESUMEN

Impacto de algunas microcistinas en el crecimiento de bacterias heterotróficas de embalses portugueses

Las microcistinas (MC) son el tipo de hepatotoxinas producidas con mayor frecuencia por cianobacterias. Los estudios han demostrado que estas toxinas afectan a varios organismos multicelulares que habitan en los ecosistemas acuáticos, sin embargo, su impacto, en las bacterias que conviven con cianobacterias de agua dulce, todavía no está claro.

En este trabajo se evaluó el impacto de las tres variantes más comunes de MC (-LR, -RR , -YR) en el crecimiento de bacterias heterótrofas aisladas de tres embalses portugueses donde a menudo se observan floraciones de cianobacterias, algunas con cepas que producen microcistina, y también en bacterias aisladas a partir de un embalse donde no se observan estos fenómenos. Para este fin, se realizó la caracterización morfológica y molecular de las cepas bacterianas que fueron después expuestas a tres concentraciones diferentes de cada variante de microcistina, y se evaluó el efecto sobre las curvas de crecimiento bacteriano.

Este estudio mostró que las microcistinas-LR, -RR y -YR pueden inducir una reducción en el crecimiento de algunas bacterias heterótrofas aisladas de aguas dulces. Hasta donde sabemos, este es el primer estudio en el que se ha evaluado el impacto de las diversas variantes de microcistinas en un grupo diverso de bacterias heterótrofas de agua dulce.

Palabras clave: *Microcistina-LR, Microcistina -RR, Microcistina -YR, bacterias heterótrofas, inhibición del crecimiento.*

INTRODUCTION

In aquatic ecosystems, phytoplankton is composed of several eukaryotic microscopic species as well as prokaryotic species such as cyanobacteria, which are photosynthetic organisms with a worldwide distribution (Castenholz & Waterbury, 1989). Cyanobacteria mainly inhabit aquatic ecosystems, where they usually co-occur with others microorganisms, such as heterotrophic bacteria (Giaramida *et al.*, 2013).

With a certain amount of nutrients and light, cyanobacteria can rapidly grow into high-density populations called blooms (Codd *et al.*, 2005). These phenomena are frequently associated with the production of toxins (Codd *et al.*, 2005); therefore, some bloom-forming cyanobacteria cause ecological, economic and health problems. Moreover, due to their overgrowth in a short time period, they may disrupt the natural balance of the aquatic system.

Heterotrophic bacteria are prokaryotes that are involved in many geochemical cycles in freshwater reservoirs, and their subsistence in aquatic ecosystems may be due to natural or anthropogenic factors, including biogeochemical processes (Figueiredo *et al.*, 2007). As a result of their role in these biogeochemical processes, bacteria are essential to the management of the aquatic ecosystem and serve as the foundation of the trophic web.

Some studies have shown that many bloom-forming cyanobacterial species prefer to grow in the presence of other bacteria (Berg *et al.*, 2009). Nevertheless, some bacteria are able to degrade cyanobacterial toxins, such as microcystins (Berg *et al.*, 2009; Giaramida *et al.*, 2013); thus, it has previously been hypothesised that heterotrophic bacteria in water may play an important role in the natural cleansing of these chemically stable hepatotoxins (Berg *et al.*, 2009). On the other hand, Giaramida *et al.* (2013) reported that exposure to microcystins has significantly contributed to the structure and microbial physiology of bacterial communities in the water bodies studied. This finding suggests a role for toxic cyanobacteria in the control of phytoplankton diversity and species abundance. However, little is known about the actual role of cyanobacteria and their interactions with heterotrophic bacteria.

Microcystins are naturally occurring cyclic peptides produced by some strains of cyanobacteria (Codd *et al.*, 2005). The most common variants of microcystins, which are also the most well studied, are microcystin-LR (MCLR), microcystin-RR (MCRR) and microcystin-YR (MCYR). The LD_{50} for MCLR in mice is 50 μg/kg (Dittmann & Wiegand, 2006). The acute lethality of MCYR is slightly lower than that of MCLR, as the LD_{50} estimate for MCYR is 70 μg/kg in mice (Dittmann & Wiegand, 2006). The LD_{50} for MCRR is approximately 10 times higher than those of the other two variants, with an estimated value of 600 μg/kg in mice (Dittmann & Wiegand, 2006).

Of the three, MCLR is the most studied variant, as it is the most representative of all microcystins (WHO, 2011). MCLR was the first microcystin chemically identified and has been associated with many incidents of toxicity involving the toxic effects of microcystins in most countries (Funari & Testai, 2008). Consequently, its toxicity is well known in animals (revised in Zegura *et al.*, 2011) and plants (Jubilee *et al.*, 2010). However, there are few studies exploring the possible effects of these cyanotoxins on heterotrophic bacteria (Christoffersen *et al.*, 2002; Dixon *et al.*, 2004; Valdor & Aboal, 2007; Yang *et al.*, 2008).

The aim of this study was to increase knowledge regarding the effects of microcystins on microbial cells. For this purpose, we evaluated the effects of the three most common microcystin variants (MCLR, MCRR and MCYR) on the growth of heterotrophic bacteria. Most of the isolates tested came from freshwater reservoirs frequently contaminated with cyanobacterial blooms and also from non-contaminated reservoirs.

MATERIALS AND METHODS

Sampling

Sampling was performed on 29 October 2012 and 29 April 2013 using 1 L sterile bottles. The first

sampling occurred at Albufeira de Magos, Açude de Monte da Barca and Albufeira de Patudos, where cyanobacterial blooms are frequently observed. The second sampling was performed at Albufeira de Castelo de Bode, a reservoir where these mass occurrences are not observed. Water samples were transported in a cooler bag in the dark to prevent both cyanobacterial growth and increases in the water temperature. More details on the characteristics of the freshwater reservoirs sampled are summarised in Table 1.

Isolation of bacteria

Bacteria were isolated from water samples from each reservoir by the plating beads method, in which 100-μL aliquots of the samples were spread using sterile glass beads. Several growth media were tested for the bacterial isolation; one of these was the non-selective Reasoner's 2A medium (R2A), which was originally designed for counting heterotrophic bacteria in drinking water samples (Reasoner & Geldreich, 1985) and is currently used to determine heterotrophic bacterial growth in water samples (Massa *et al.*, 1998). The other media used were Lysogeny Broth (LB) medium, a rich medium used for bacterial growth, and Z8 medium, which is formulated for cyanobacterial growth (Skulberg & Skulberg, 1990). The latter medium was used to determine whether the bacteria that co-occur with cyanobacteria would also be able to use the same nutrients to grow.

All the inoculated plates were incubated for four days at $20 \pm 2\,^{\circ}C$ in the dark to prevent cyanobacterial growth. No bacterial growth was observed on the Z8 medium. After the incubation period, two to three different individual colonies were selected from each sample incubated at $20 \pm 2\,^{\circ}C$ from the R2A and LB media. In total, thirteen purified colonies were obtained in Nutrient Agar (NA) medium.

Characterisation and molecular identification of the isolates

The bacterial shape was assessed microscopically. To classify the isolates as gram-positive or gram-negative, microscope slides of cell suspensions from each isolate were prepared in the automated PREVI® Color Gram system (Biomeriux, USA).

Bacterial DNA extraction was performed by two different methods. For gram-negative bacteria, we used the boiling method, whereas for gram-positive bacteria, we employed the Invisorb® Spin Plant Mini Kit (INVITEK), following the manufacturer's instructions.

With regard to the boiling method, a loopful of each bacterial culture was washed twice, resuspended in 300 μL apyrogenic water, subjected to boiling at 100 °C for 15 min in a water bath and centrifuged at 10 000 rpm for 5 min. The supernatants were recovered and stored at −20 °C until use.

The concentration and purity of nucleic acids were assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Table 1. Characteristics of the freshwater reservoirs sampled (SNIRH, 2011). *Características de los embalses de agua dulce muestreados (SNIRH, 2011).*

	Monte da Barca	Magos	Patudos	Castelo do Bode
Location	Coruche	Salvaterra de Magos	Alpiarça	Tomar
Hydrographic basin	Tejo	Tejo	Tejo	Tejo
Usage	Recreation	Irrigation	Recreation	Drinking water supply; Energy production; Recreation; Flood control
Full capacity hm^3	—	3.03	0.33	1095
Average chlorophyll-*a* ($\mu g\ L^{-1}$)*	—	34.0	252.9	1.8
Trophic state	—	Eutrophic	Eutrophic	Oligotrophic

— Information not available.

* The average chlorophyll-*a* values presented are means from January to November; data provided by APA (Agência Portuguesa do Ambiente).

Aliquots of 2 μL (100 ng) of template DNA were used for PCR amplification of the 16S rRNA gene. PCR was performed in 25 μL reaction mixtures containing 1× PCR buffer (Invitrogen), 0.05 mM dNTPs, 1 μM of each primer, 1 mg/mL BSA, 3 mM $MgCl_2$ (Invitrogen) and 1 U of Taq polymerase (Invitrogen).

Two sets of universal bacterial primers were used. The pair 104F and 907R (Chaves, 2005) amplified a fragment of ca. 800 bp. The other primer pair, 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Turner *et al.*, 1999), yielded an amplicon of ca. 1480 bp. The reactions were performed in a

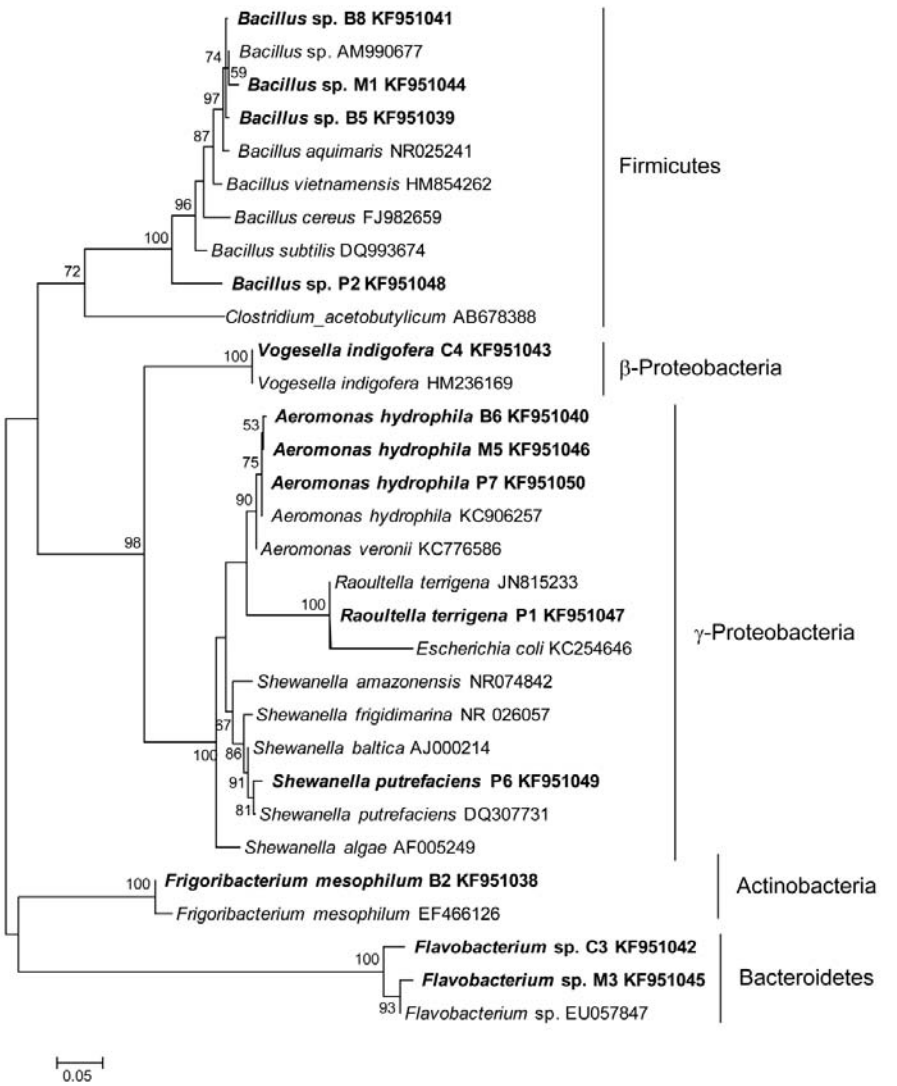

Figure 1. Phylogenetic tree of isolates from this study and related taxa retrieved from GenBank, obtained by Maximum Likelihood analysis of 16S rRNA gene sequences. The percentage bootstrap values of 1000 replicates are given at each node, and only values above 50 are displayed. GenBank accession numbers are indicated after the species designation. Names in boldface correspond to sequences determined in this study. *Árbol filogenético de los aislamientos de este estudio y taxones relacionados obtenidos de GenBank, a partir del análisis de máxima verosimilitud de secuencias del gen 16S rRNA. Los valores de arranque porcentuales de 1000 repeticiones se dan en cada nodo, sólo se muestran los valores superiores a 50. Los números de acceso a GenBank se indican después de la designación de especies. Los nombres en negrita corresponden a secuencias determinadas en este estudio.*

Tpersonal thermocycler (Biometra®) with a hot lid (95 °C) using the following conditions: an initial denaturation at 94 °C for 5 min. followed by 40 cycles of 94 °C for 1 min, annealing at 48-52 °C (variable depending on the isolate) for 1 min, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. The PCR products were resolved by electrophoresis through 1% (w/v) agarose gels at 75 V for 45 min in 1× TAE buffer. GelRed was incorporated in the gel to allow the visualisation of the PCR amplicons. The gel image was acquired using a gel transilluminator (UVITEC).

The PCR products were purified with the peqGOLD Cycle-Pure Kit (peqLab). Some bacterial isolates yielded nonspecific PCR products, which were eliminated by extracting the amplicons of interest from the gel and purifying them with NucleoSpin® Gel and PCR clean-up kits (MACHEREY-NAGEL), according to the manufacturer's instructions. The samples were sent to the UTI-INSA facilities for sequencing in both directions on an ABI 3130xl Genetic Analyzer using BigDye terminators and the same primers used for amplification. Standard protocols were followed.

The chromatograms from the sequencer were read and edited using the BioEdit program (Hall, 1999). The sequences were analysed with the GenBank nucleotide data library using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the reliability of the 16S rRNA gene sequences and determine their closest relatives.

Bacterial 16S rRNA gene sequences were checked for PCR chimeras using DECIPHER's Find Chimeras web tool (Wright *et al.*, 2012) and were submitted to the GenBank database under accession numbers KF951038-KF951050. Partial 16S rRNA gene sequences were aligned with MUSCLE (Edgar, 2004) using MEGA6 (Tamura *et al.*, 2013). A total of 562 nucleotide positions were present in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The phylogenetic tree presented was constructed using the Maximum-Likelihood (ML) method with the Tamura–Nei substitution model and partial deletion treatment. Node support was estimated using 1000 bootstrap replicates.

Bacterial cell growth

The pre-inoculums were prepared in 100 mL Erlenmeyer flasks containing 10 mL NB medium. The cells were incubated overnight at 20 °C on an orbital shaker (SO3) at 300 rpm.

Bacterial growth was assessed in 96-well flat-bottomed microplates. Five replicates of each isolate were inoculated in 200 µL Nutrient Broth (NB) medium with an initial optical density of 0.05. Wells containing bacterial suspension without toxin (growth control) were also included. Extracts, purified according to Dias *et al.* (2009), from each of the three microcystin variants (MCLR from LMECYA 110, MCRR from LMECYA 103, and MCYR from LMECYA 179) were inoculated individually at final concentrations of 1 nM, 10 nM and 1000 nM. These MC concentrations are commonly found in the freshwater reservoirs sampled. The highest MCYR concentration used was 300 nM rather than 1000 nM because of stock limitation. The cultures were incubated under the same conditions as the pre-inoculums.

The optical densities of each microplate were measured every 30 min at 600 nm for approximately 12 hours using a Multiskan Ascent Thermo Labsystems microplate reader after fast shaking for 15 sec.

Growth curves of each tested isolate were determined, and the results were expressed as the means ± SE of five replicates. All data were evaluated by F-test and subsequently with Student's t-test for assessing the statistical significance ($p < 0.05$) of the difference between the growth from cultures exposed to toxins and the control group (not exposed to MCs).

RESULTS

Characterisation of the heterotrophic bacteria isolated

A code including letters and numbers was attributed to the isolates (B-from Açude de Monte da Barca; M-Albufeira de Magos; P-Albufeira de Patudos; C-Albufeira de Castelo de Bode). We were able to recover 13 isolates: four from Monte

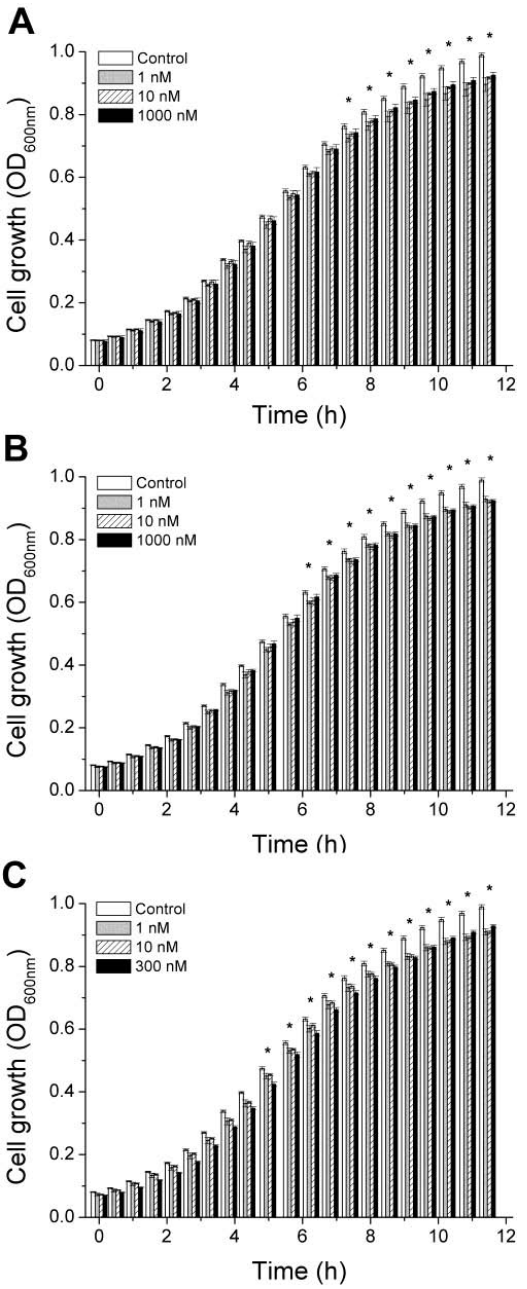

Figure 2. Growth curves of *Aeromonas hydrophyla* B6 in NB medium and in the presence of A) MCLR; B) MCRR; and C) MCYR at the concentrations identified in the graphs. Each point represents the mean of $OD_{600\ nm} \pm SE$ from five replicates; * significant differences with respect to the control ($p < 0.05$). *Curvas de crecimiento de* Aeromonas hydrophyla *B6 en medio NB y en presencia de diferentes concentraciones de A) MCLR, B) MCRR y C) MCYR, como se indica en los gráficos. Cada punto representa la media de* $DO_{600\ nm} \pm SE$ *de cinco réplicas; * diferencias significativas en relación con el control de (*$p < 0.05$*).*

da Barca, three from Magos, four from Patudos and two from Castelo de Bode.

These isolates were identified by their 16S rRNA phylogenetic positioning as displayed in Fig. 1. They were distributed among five phyla: four Firmicutes (*Bacillus* sp.), one Actinobacteria (*Frigoribacterium mesophilum*), two Bacteroidetes (*Flavobacterium* sp.), one β-proteobacteria (*Vogesella indigofera*), and five γ-proteobacteria (one *Raoultella terrigena*, three *Aeromonas hydrophila*, and one *Shewanella putrefaciens*).

Effects of microcystins on bacterial growth

During the growth experiments, it was verified that some isolates (B2, B5, B8, M3, P2, P7 and C3) were unable to grow in liquid medium (neither NB nor LB). Therefore, it was not possible to proceed with the subsequent assays for these isolates.

The growth curves of isolates B6, M1, M5, P1 and P6 in the presence of serial dilutions of MCLR, MCRR and MCYR are presented next.

The results obtained for *Aeromonas hydrophyla* B6, from Monte da Barca reservoir, in the presence of each of the three MCs variants are quite similar (Fig. 2). There was an inhibition of the growth for all the variants and concentrations tested. Particularly, after 7 h of growth, all the differences were significant with respect to the control ($p < 0.05$).

The growth curves of *Bacillus* sp. M1 and *Aeromonas hydrophyla* M5, isolated from Magos reservoir, are depicted in Figure 3. The effects induced by the three MC variants were quite similar in the M1 isolate, which showed slight growth inhibition that was only significant at the end of the assays (Fig. 3 A-C). There were almost no differences between the control and isolate M5, which had some growth problems because it did not reach a high optical density (Fig. 3 D-F).

There were no major differences between the exposure to MCLR or MCRR that inhibited the growth of *Raoultella terrigena* P1 (Fig. 4A and B) and *Shewanella putrefaciens* P6 (Fig. 4D and E), which were recovered from Patudos reservoir. However, the effects of MCYR appeared less

pronounced (Fig. 4C and F). After 10 h of growth, all the differences were significant with respect to the control ($p < 0.05$).

Vogesella indigofera C4, isolated from Castelo de Bode (the non-contaminated reservoir), showed no differences from the control because the growth of this isolate was not affected by the presence of microcystins (data not shown).

We have also determined whether the use of different initial optical densities (0.02, 0.05 and 0.1) would yield a different outcome; however, we did not observe any differences.

DISCUSSION

Cyanobacteria and heterotrophic bacteria are important parts of aquatic ecosystems; thus, studies that examine the interactions of both organisms to elucidate the relationships that occur in aquatic ecosystems are of major importance.

In this work, we have recovered thirteen bacterial isolates from freshwater reservoirs with frequent occurrence of cyanobacteria and also from a reservoir where these phenomena do not occur. The isolates represented a wide range of hetero-

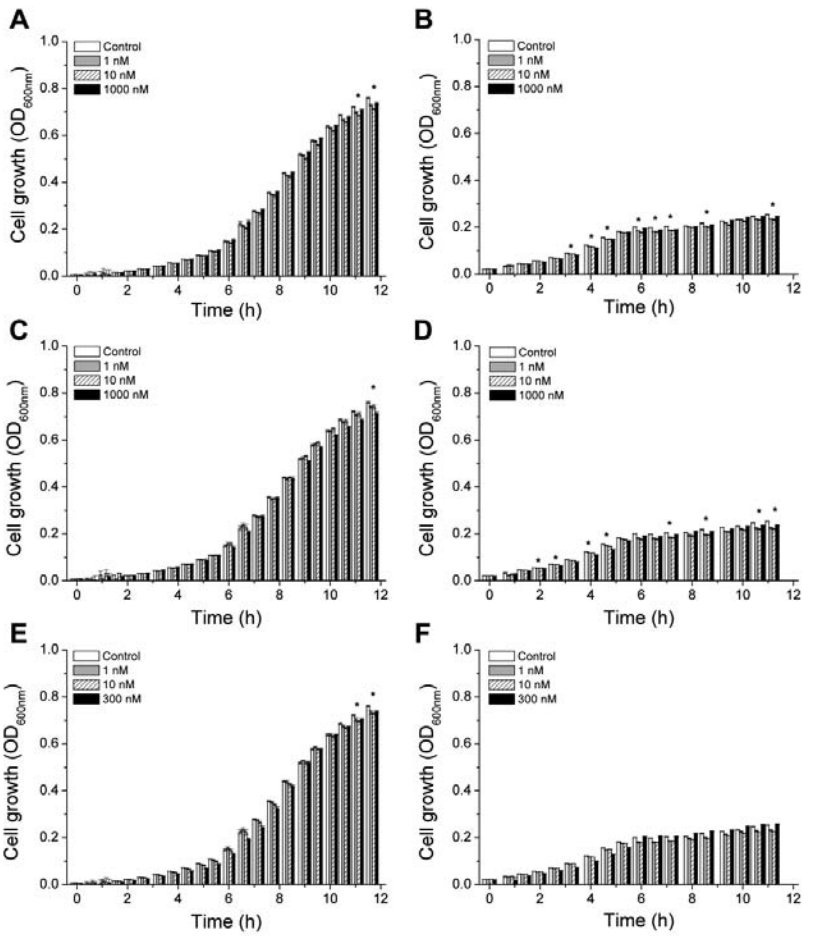

Figure 3. Growth curves of *Bacillus* sp. M1 (A, C, E) and *Aeromonas hydrophyla* M5 (B, D, F) in NB medium and in the presence of A, B) MCLR; C, D) MCRR; and E, F) MCYR at the concentrations identified in the graphs. Each point represents the mean of $OD_{600\,nm}$ ± SE from five replicates; * significant differences with respect to the control ($p < 0.05$). *Curvas de crecimiento de* Bacillus *sp. M1 (A,C,E) y M5* Aeromonas hydrophyla *(B, D, F) en medio NB y en presencia de diferentes concentraciones de A, B) MCLR; C, D) MCRR y E, F) MCYR, como se indica en los gráficos. Cada punto representa la media de* $DO_{600\,nm}$ ± *SE de cinco réplicas; * diferencias significativas en relación con el control de* $p < 0.05$.

trophic bacteria distributed in five different phyla: Firmicutes, Actinobacteria, Bacteroidetes, β-Proteobacteria and γ-Proteobacteria. The diversity and type of heterotrophic bacteria isolated in this study agree with what has been found previously in habitats where these bacteria cohabitate with cyanobacteria (Eiler & Bertilsson 2004; Berg *et al.*, 2009; Parveen *et al.*, 2013). We observed that the majority of the bacteria belonged to *Bacillus*, *Aeromonas* and *Flavobacterium* genera, which is consistent with previous studies (Berg *et al*., 2009).

It is known that microcystins cross animal cell membranes through the transmembrane solute carriers transport family OAPT (Organic Anion Polypeptide Transporters) (Hagenbuch & Meier, 2004; Fischer *et al.*, 2005). Regarding bacteria, it has been shown that microcystins can permeate the membranes of both gram-negative and gram-positive bacteria (Dixon *et al.*, 2004; Yang *et al.*, 2010). Therefore, we can presume that MCs might enter bacterial cells and provoke cell damage.

The growth curves for the isolates tested showed significant differences between the con-

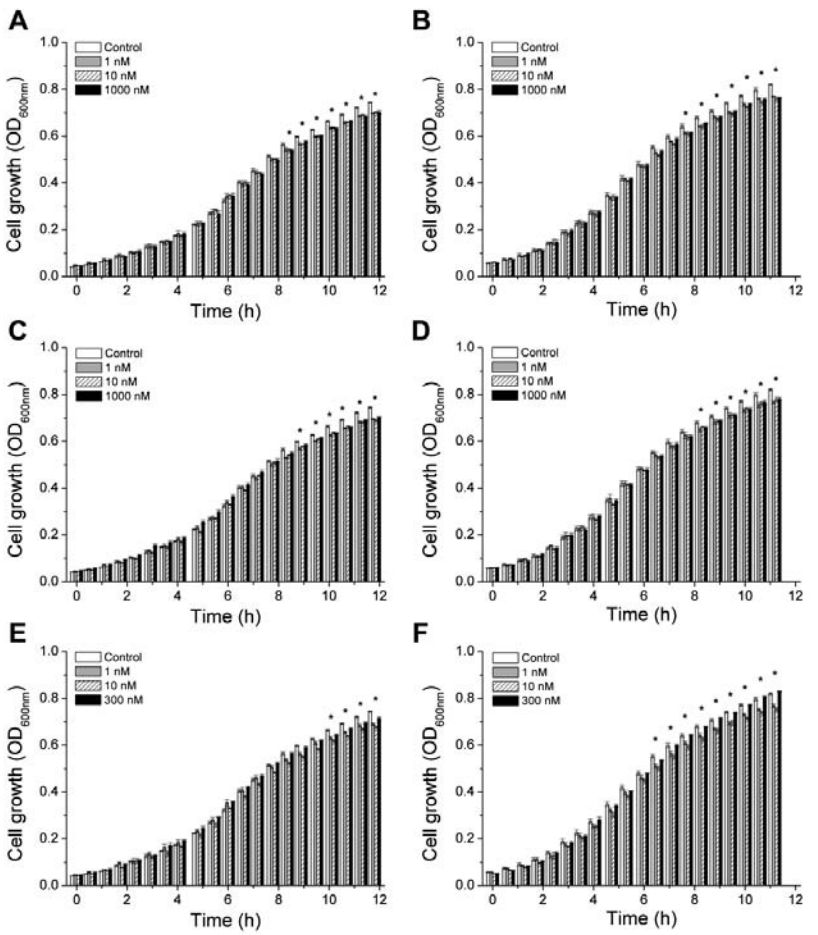

Figure 4. Growth curves of *Raoultella terrigena* P1 (A, C, E) and *Shewanella putrefaciens* P6 (B, D, F) in NB medium and in the presence of A, B) MCLR; C, D) MCRR; and E, F) MCYR at the concentrations identified in the graphs. Each point represents the mean of $OD_{600\ nm}$ ± SE from five replicates; * significant differences with respect to the control ($p < 0.05$). MCYR at 300 nM did not cause significant differences in P6 growth (F) with respect to the control. *Curvas de crecimiento de* Raoultella terrigena *P1 y* Shewanella putrefaciens *P6 en medio NB y en presencia de diferentes concentraciones de A, B) MCLR; C, D) MCRR y E, F) MCYR, como se indica en los gráficos. Cada punto representa la media de* $DO_{600\ nm}$ ± *SE de cinco replicas; * diferencias significativas en relación con el control de* p < *0.05. El 300 nM de MCYR en el crecimiento P6 (F) no presentó diferencias significativas respecto al control.*

trol cells and the microcystin-treated cells only after they reached late exponential/stationary phase. However, it was found that MCs reduced the growth of most bacteria tested (4 out of 6). Yang *et al.* (2008) observed that *E. coli* only showed growth inhibition in the initial growth phase when cells were treated with MCRR (1000, 5000, 10 000 and 15 000 nM). The normal growth rate was re-established and the growth curves of treated and untreated bacteria became parallel, only showing a reduction in growth when exposed to 1000 and 5000 nM concentrations and displaying a marked inhibition only for the two higher concentrations tested (Yang *et al.*, 2008). In this study, MCLR, MCRR and MCYR reduced, but did not inhibit, bacterial growth. Nevertheless, it should be noted that all growth experiments presented here were performed with MC concentrations closest to the lowest concentration tested by Yang *et al.* (2008), which also did not cause marked differences in *E. coli* growth curves. As mentioned previously, these concentrations were selected because they are similar to the concentrations found in freshwater reservoirs; thus, they provide a more realistic idea of what can naturally occur.

The growth of *Aeromonas hydrophyla* M5 and *Vogesella indigofera* C4 was not affected by the presence of MCs. We presume that this finding is not directly related to their type of cell wall, as other gram-negative isolates tested were affected, or to their taxa, as the growth of other *Aeromonas hydrophyla* tested (B6) was inhibited. Therefore, one can only hypothesise that these isolates may have features that prevent them from being affected by MC. The isolate *Vogesella indigofera* C4 was recovered from a freshwater reservoir where it is not usual to observe cyanobacterial blooms, and its growth was not affected by the presence of microcystins. In fact, we did not observe significant differences in the growth of MC-treated cells between bacteria that co-occur with cyanobacterial toxins and bacteria that do not inhabit this type of ecosystem. This suggests that although some heterotrophic bacteria are frequently in contact with MCs, they did not reveal a genetic adaptation to become MC resistant.

Furthermore, some strains of bacteria are able to degrade cyanobacterial toxins (Berg *et al.*, 2009; Giaramida *et al.*, 2013), especially *Flavobacterium* (Berg *et al.*, 2009). It would be interesting to determine whether the lack of a microcystin-dependent effect on the growth of these two isolates could be related to their ability to degrade these toxins.

Thus far, minimal progress has been made toward elucidating the possible effects of microcystins on microbes that play an important role in the aquatic ecosystem. In fact, to our knowledge, this is the first study in which the impact of several variants of microcystins was evaluated in a more diverse set of freshwater heterotrophic bacteria. Although we observed growth inhibition induced by MCs, the minor effect on bacterial growth led us to propose several hypotheses that require further elucidation.

Author's contributions

EV conceived and designed the research, DM performed the experiments under the supervision of EV, DM and EV analysed the data, and EV wrote the paper with advice and consent from all authors.

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